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7-Methylpterin and 7-Methyllumizine: Oxidative Degradation Products of 7-Methyl-Substituted Pteridines in Methanogenic Bacteria

ROBERT H. WHITE

Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

Received 21 September 1984/Accepted 5 February 1985

7-Methylpterin and 7-methyllumizine were isolated and identified in extracts of methanogenic bacteria which had been extracted in air with ethanol-water. Ethanol-water preparations of cells extracted under nitrogen or hydrogen were devoid of these compounds. Extracts of cells obtained in the presence of air also had an increased amount of a complex arylamine which, on acid hydrolysis, gave 1 mol each of phosphate, 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane, and α-hydroxyglutaric acid. Gas chromatography-mass spectrometry was used to identify the 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane as its tetratrimethylsilyl derivative and the α-hydroxyglutaric acid as the *n*-butyl ester derivative of its γ-lactone. When exposed to air, extracts of cells prepared in the absence of air produced 6-acetyl-7-methylpterin and 7-methylxanthopterin in addition to 7-methylpterin and 7-methyllumizine. It is concluded that these compounds are derived from the oxidative cleavage of the tetrahydromethanopterin, which is present in these bacteria, by a series of reactions analogous to those known to occur in the oxidative cleavage of tetrahydrofolic acid.

6-Substituted pterins and their tetrahydro derivatives occur widely in bacteria (2). The most important of these are the folic acids and their polyglutamate derivatives, which are known to be the principal coenzymes involved in the metabolism of C_1 compounds (2). Other 6-substituted pterins known to occur in bacteria include (2-amino-4-hydroxy-6-pteridinyl)glycerol phosphate (9), neopterin 2′:3′-phosphate (32), 6-carboxypterin, 6-methylpterin and 6-hydroxymethylpterin (13, 31), and the pterin portion of the molybdenum cofactor (10). Recent reports have shown that both 6-substituted and 6-unsubstituted pterins with a 7-methyl group occur in methanogenic bacteria (11, 12, 26, 33, 34) which are members of the *Archaeobacteria* (36). If these 7-substituted pterins are found to occur only in methanogenic bacteria, then they will be useful as specific biochemical markers for this group of bacteria. If, on the other hand, all members of the *Archaeobacteria* are found to contain these 7-substituted pterins, then these pterins could be used to identify *Archaeobacteria*. It is also possible that these 7-methyl-substituted pterins could arise biosynthetically by a route(s) distinctively different from that used for the biosynthesis of the normal 7-unmethylated pterins. If the biosynthetic route for pterins in the *Archaeobacteria* is different from that found in the *Eubacteria*, it would represent a fundamental metabolic difference between these primary kingdoms (36). To explore these possibilities, I have investigated the reported occurrence of 7-methylpterin in methanogenic bacteria. The results of this work clearly show that this compound is not present in the bacteria but is derived by the oxidative cleavage of the tetrahydromethanopterin present in the cells during their extraction in the presence of air.

MATERIALS AND METHODS

Growth of organisms. Frozen cells of *Methanobacterium formicicum*, grown with formate as the sole energy source and harvested with a continuous-flow centrifuge as previously described (24), were supplied by J. G. Ferry, Virginia Polytechnic Institute and State University, Blacksburg. Rumen strain 10-16B was grown in 2-liter bottles containing 500 ml of the medium described by Lovley et al. (18) under an atmosphere of H_2–CO_2 (80/20) at 30 lb/in^2. *Methanobacterium thermoautotrophicum* was grown in 100-ml glass bottles containing 20 ml of the medium described by Schönheit et al. (27). *Methanosarcina* sp. strain TM-1 (38) was grown as previously described (19) and was supplied by D. R. Lovley (Virginia Polytechnic Institute and State University) as frozen cells.

Extraction and purification of pteridines. Cells were removed from the anaerobic growth medium by centrifugation in either the presence or the absence of air. For each gram (wet weight) of cell pellet, 1 ml of water and 4 ml of 100% ethanol was added. The cells were suspended by shaking, and the resulting mixture was heated at 80°C for 1 h. After centrifugation and removal of the clear liquid extract, the resulting pellet was reextracted with 5 ml of 60% ethanol and heated at 80°C for 10 min. The combined extracts were concentrated to dryness, placed in 60% ethanol, and purified as described below. Extractions in the absence of air were done with solutions degassed with nitrogen or hydrogen. Thin-layer chromatography (TLC) separations were effected on either Avicel (Analtech, Inc., Newark, Del.) cellulose plates with 2-butanol–formic acid–water (6:1:2, vol/vol/vol) or silica gel 60 F-254 (E. Merck AG, Darmstadt, Germany) TLC plates with *n*-butanol–acetic acid–water (12:3:5, vol/vol/vol) or the top layer of *n*-butanol–acetic acid–water (5:1:4, vol/vol/vol). These are hereafter referred to as chromatographic systems I, II, and III, respectively.

Spectroscopy. UV-visible spectra were obtained with a Varian-Cary 219 spectrophotometer. Fluorescent absorption and emission spectra were obtained on a Perkin-Elmer 650-40 fluorescence spectrophotometer.

Gas chromatography-mass spectrometry. The pterins and lumizines were purified by preparative TLC on Avicel plates. The individual spots were visualized by their fluorescence under UV light, and the areas containing the desired

bands were removed and eluted with methanol. After evaporation of the methanol, the ditrimethylsilyl derivatives were prepared by heating the samples with 20 μl of a 1:1 mixture of pyridine and *N,O*-bis-(trimethylsilyl)acetamide at 100°C for 10 min. The derivatives were separated by gas chromatography in a glass column (1/8 inch by 3 feet [ca. 0.32 by 91.4 cm]) containing 3% OV-1 and programmed from 100°C at 10 min.

Synthesis of 7-methylpterin, 7-methyllumizine, and 7-methylxanthopterin. 7-Methylpterin was prepared by the condensation of 2,4,5-triamino-6-hydroxypyrimidine with glycolaldehyde basically as described by Mowat et al. (21). Hydrolysis of the 7-methylpterin to 7-methyllumizine was effected by 6 M HCl hydrolysis at 100°C for 30 h (30). 7-Methylxanthopterin was prepared by the condensation of 2,4,5-triamino-6-hydroxypyrimidine sulfate with diethyl oxalacetate as described by Elion et al. (5).

Permanganate oxidations. Alkaline permanganate oxidation was performed as described by Forrest and Mitchell (8). Excess 0.01 M $KMnO_4$ was added to the samples in 0.1 M NaOH, and the resulting solution was heated at 100°C for 15 min. Excess ethanol was added to destroy the remaining $KMnO_4$ and, after neutralization with HOAc and centrifugation, TLC was run on the resulting clear solution.

Assay, purification, and analysis of the arylamine. The arylamine present on TLC plates was readily identified as an orange-red band after the plates were sprayed with a 1% solution of 1,2-naphthoquionine-4-sulfonate in 5% acetic acid (29). The compound had an R_f of 0.15 with chromatographic system I and an R_f of 0.05 with system II. The compound was purified to homogeneity by preparative TLC in chromatographic system I, followed by preparative TLC in system II. The resulting material had a maximum absorbance (λ_{max}) at 268 nm at pH 3.5 and, after hydrolysis for 12 h with 6 M HCl, released P_i, which was assayed by the method of Chen et al. (4). Treatment of a portion of the sample with 3 M HCl in *n*-butanol for 4 h at 90°C and subsequent evaporation of the solvent produced the *n*-butyl ester of the γ-lactone of α-hydroxyglutaric acid, which was identified by comparing its gas chromatographic retention time and mass spectrum with that of a known sample. Acid hydrolysis, 1 M HCl at 100°C for 1 h, quantitatively hydrolyzed the arylamine to 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane, which had an R_f of 0.38 in chromatographic system I and an R_f of 0.25 in system II. A sample of this 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane gave a single gas chromatographic peak as the tetratrimethylsilyl derivative. The derivative was prepared by allowing a dried sample of the compound to react with a mixture of chlorotrimethylsilane-hexamethyldisilane-pyridine (1:3:9, vol/vol/vol) for 1 h at room temperature. The mass spectrum of this compound showed major ions at M^+ 515 (<1%), 427 (1%), 320 (12%), 221 (100%), 208 (32%), 205 (30%), and 106 (78%), which is identical to the mass spectrum observed for the tetratrimethylsilyl derivative of a synthetic sample of 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane.

Quantitation of the arylamine was accomplished by measuring the amount of orange color formed from the reaction between the arylamine and 1,2-naphthoquionine-4-sulfonate in 5% acetic acid. The assay consisted of allowing 10 to 30 μl of an aqueous solution of the sample to react with 50 μl of 20 mM 1,2-naphthoquionine-4-sulfonate in 5% acetic acid for 10 min at room temperature, diluting with water to 1 ml, and reading the absorbance at 485 nm. *N*-Aminobenzyl-L-glutamate was used as a standard and gave a linear standard curve of from 25 to 150 nmol.

RESULTS

Chromatographic separation of extracts from *M. formicicum*, *M. thermoautotrophicum*, *Methanosarcina* sp. strain TM-1, and rumen strain 10-16B extracted in the presence of air in chromatographic system II all showed a large number of highly fluorescent compounds. On the basis of the comparison of the R_fs of these compounds with knowns, their change in R_f on acid hydrolysis, and their absorption and fluorescence spectra, many of these fluorescent bands were shown to result from combinations of the flavin adenine dinucleotide, flavin mononucleotide, coenzyme F_{420} or an isomer of F_{420}, and methanopterin present in the extract. Most of these compounds have previously been reported as occurring in methanogenic bacteria (15, 20, 25, 33, 34). Among the many spots observed, two with exceptionally strong fluorescence had R_fs higher than any of the above compounds. These, as well as several of the other fluorescent compounds, were not detected in extracts generated in the absence of air. The faster-moving spot (R_f = 0.44) gave a green fluorescence with long-wavelength UV light and was seen in extracts of strain 10-16B and *M. formicicum*. The slower-moving spot (R_f = 0.35), when viewed under the same conditions, gave a blue fluorescence and was detected in all organisms except *M. formicicum*. These green and blue fluorescent spots had R_fs of 0.33 and 0.44, respectively, in chromatographic system I and 0.46 and 0.53, respectively, in system III. The faster-moving compound was consistently the green fluorescent spot, and the slower-moving compound was always the blue fluorescent spot. The green fluorescent spot, after elution from the plate with methanol, had a λ_{max} at 245 and 324 nm in 0.01 M phosphate buffer (pH 7.0) or in dilute acetic acid and a λ_{max} at 252 and 358 nm in 0.1 M sodium hydroxide. The blue fluorescent spot had a λ_{max} at 271 and 338 nm in 0.01 M phosphate buffer and 250 and 357 nm in 0.1 M sodium hydroxide. After these absorption data were compared with absorption spectra previously published for 7-methylpterin (12, 21) and after the changes in the excitation and emission spectra of these compounds were considered as a function of pH with known compounds, the higher-R_f compound was identified as 7-methyllumizine and the lower-R_f compound was identified as 7-methylpterin. The fluorescence data for the known and unknown compounds were identical (Table 1). The known and unknown compounds comigrated in all of the above TLC systems and were separable from the 6-methyl isomers in chromatographic system II. During gas chromatography, the ditrimethylsilyl derivatives of the knowns and unknowns coeluted, and each gave the expected mass spectrum for its

TABLE 1. Spectroscopic properties of isolated pteridines[a]

Pteridine	Wavelength (nm)	
	Excitation	Emission
7-Methyllumizine		
0.01 M PO_4^{2-} (pH 7.0)	337	460
0.1 M HCl	337	375,473
0.1 M NaOH	368	455
7-Methylpterin		
0.01 M PO_4^{2-} (pH 7.0)	357	434
0.1 M HCl	None	None
0.1 M NaOH	364	446

[a] Pteridines were chromatographed on silica gel with chromatographic system II, eluted from the TLC plate with methanol, and after evaporation of the methanol, dissolved in the indicated solution.

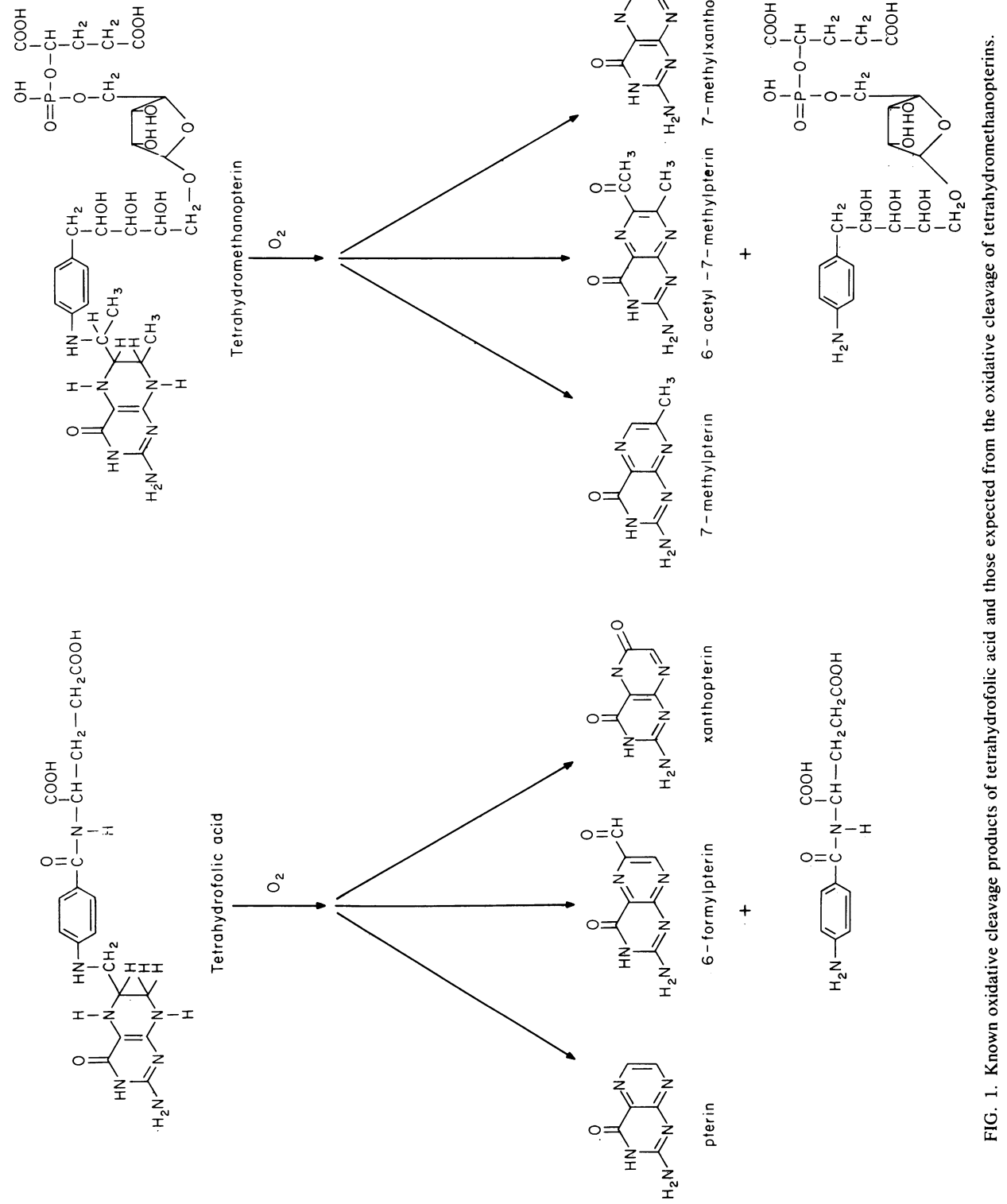

FIG. 1. Known oxidative cleavage products of tetrahydrofolic acid and those expected from the oxidative cleavage of tetrahydromethanopterins.

ditrimethylsilyl derivative; i.e., the 7-methylpterin had intense ions at m/z 306 ($M^+ - 15$), 100% and m/z 321 M^+, 66% and the 7-methyllumizine had intense ions at m/z 307 ($M^+ - 15$), 28%; m/z 322 M^+, 46%; and m/z 73, 100%. These mass spectra are very similar to those reported for the ditrimethylsilyl derivative of 6-methylpterin (14, 17).

The higher-R_f compound was found to be stable to 6 M HCl hydrolysis at 110°C for 24 h and to treatment with $NaIO_4$. Stability to acid hydrolysis and the inability to be cleaved by periodate was also observed for the known sample. Each unknown was oxidized with $KMnO_4$ in 0.01 M NaOH to the corresponding 7-carboxylic acid, which was separable from the corresponding pteridine-6-carboxylic acid in chromatographic system II.

Extracts of strain TM-1 prepared in the absence of air developed three additional intense-blue fluorescent spots when exposed to air. The lower spot (R_f = 0.33; chromatographic system II) was identified as 7-methylpterin as described above. The next-higher spot (R_f = 0.4; chromatographic system II) comigrated with 7-methylxanthopterin in all the above chromatographic systems and gave an absorption spectrum identical to that of a known sample of 7-methylxanthopterin; i.e., in 0.01 M phosphate buffer (pH 7.0), the λ_{max} was 277 and 382 nm; in 0.1 M HCl, the λ_{max} was 265 and 357 nm; and in 0.1 M NaOH, the λ_{max} was 253, 272, and 383 nm. This is in agreement with values found in the literature (21). The highest-R_f spot (R_f = 0.59; chromatographic system II) had absorption maxima at 277 and 355 nm in 0.01 M phosphate buffer, 260 and 338 nm in 0.1 M HCl, and 251, 311, and 360 nm in 0.1 M NaOH. On the basis of a comparison of these data with those published for 6-formylpterin (35), this compound has been tentatively identified as 6-acetyl-7-methylpterin.

TLC plates run with ethanol extracts and developed in chromatographic system II showed a single, intense red-orange band (R_f = 0.049) when sprayed with 1,2-naphthoquionine-4-sulfonate, indicating an arylamine. Quantitation of the arylamine in strain 10-16B showed 20.1 nmol/mg (dry weight) when extracted in the presence of air and 17.5 nmol/mg (dry weight) when extracted in the absence of air. Strain TM-1 extracted in the absence of air contained 8.53 nmol/mg (dry weight). The compound purified from strain 10-16B contained 1 mol of phosphate, had a λ_{max} of 260 nm at pH 3.5, and contained α-hydroxyglutaric acid and 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane. Quantitation of the moles of phosphate in the arylamine was based on an ε_{max} at 258 nm of 235 M^{-1} cm^{-1} at pH 3.5 for the 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane cation. This is in agreement with the measured ε_{max} at 258 nm of 224 M^{-1} cm^{-1} for *p*-aminophenylacetic acid in 0.05 M HCl.

DISCUSSION

Dihydrofolates and tetrahydrofolates are known to readily undergo air oxidation, resulting in the separation of the pterin from the *p*-aminobenzoic acid portion of the molecule (2, 23). The major products of this cleavage have been identified as *p*-aminobenzoylglutamate and a series of pterins including pterin, dihydropterin, xanthopterin, and 6-formylpterin (1, 2, 23) (Fig. 1). Under suitable conditions, the production of *p*-aminobenzoylglutamate can be quantitative and can form the basis of a quantitative method for determining tetrahydrofolates (7, 37). Considering the structural similarities between methanopterin (34) and folic acid (2) and that tetrahydromethanopterin has recently been shown to be the active form of methanopterin in methanogenic bacteria (6), an oxidative cleavage reaction similar to that observed for tetrahydrofolic acid would be expected to occur when extracts of cells containing this compound are exposed to air (Fig. 1). However, unlike the oxidative cleavage of tetrahydrofolate, which is known to produce nonmethylated pterins, the oxidative cleavage of tetrahydromethanopterin should produce 7-methyl-substituted pterins (Fig. 1). In addition, the arylamine fragment produced from tetrahydromethanopterin would be quite different from that produced by the cleavage of tetrahydrofolic acid as outlined in Fig. 1.

The identification of 7-methylpterin in only those extracts generated in the presence of air verifies that an analogous oxidative cleavage reaction is occurring in these samples. 7-Methylpterin was found in strain 10-16B at a level of 3.6 nmol/mg (dry weight) of cells. On exposure to air, extracts of strain TM-1 were found to produce 7-methylxanthopterin and 6-acetyl-7-methylpterin in addition to 7-methylpterin. Each of these products is expected to result from the oxidative cleavage of tetrahydromethanopterin based on products identified from the oxidative cleavage of tetrahydrofolate, i.e., pterin, xanthopterin, and 6-acetylpterin (23).

In addition to producing the above-described 7-methylpterins, the cleavage of tetrahydromethanopterin would also be expected to generate the arylamine shown in Fig. 1. Quantitation of the arylamine, however, is complicated, since even extracts of cells generated in the absence of air were found to contain this compound. Its abundance in extracts of strain 10-16B increased from 17.5 nmol/mg (dry weight) in cells extracted in the absence of air to 20.1 nmol/mg (dry weight) in cells extracted in the presence of air. The difference in these values of 2.6 nmol/mg (dry weight) is very close to the amount of 7-methylpterin isolated from the cells, which adds further support to the idea that the 7-methylpterins are generated by the oxidative cleavage of tetrahydromethanopterin.

The arylamine assayed above was found to contain α-hydroxyglutaric acid, phosphate, and 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane, indicating that the arylamine was, in fact, the cleavage fragment shown in Fig. 1. The occurrence of this compound, even in extracts of methanogenic bacteria not exposed to air, has important implications for the biosynthesis of methanopterin, since this arylamine could condense directly with the pyrophosphate ester of dihydro-6-αhydroxyethyl-7-methylpterin to produce dihydromethanopterin. The dihydromethanopterin could then be reduced to tetrahydromethanopterin. These proposed reactions are analogous to the condensation of the pyrophosphate ester of 7,8-dihydro-6-hydroxymethylpterin with *p*-aminobenzyl glutamate, a major step in the biosynthesis of folic acid (3, 22, 28).

Since lumizine (2,4-dihydroxypteridine) is not a known product in the oxidative cleavage of tetrahydrofolates (23), the exact origin of the 7-methyllumizine is not clear. It may originate from a methanopterin-like molecule which contains a 7-methyllumizine in place of the 7-methylpterin. The enzymatic replacement of the amino group of pterins for a hydroxyl group by a bacterial enzyme has been described previously (16) and might be related.

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